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RESEARCH PROJECT INITIATION

Date: May 13, 1974

Project Title: Synthesis of Elastase Inhibitors

Project No: G-33-675

Principal Investigator: Dr. James C. Powers

Sponsor: Public Health Service, NIH

Agreement Period: From April 1, 1974 Until June 30, 1975

Type Agreement: Contract No. N01 HR 42939

Amount: $28,000

Reports Required: Progress Report, Final Report, Manpower Report.

Sponsor Contact Person (s):

Contractual
Thru ORA
Mr. G. A. Campion
National Institutes of Health
National Heart and Lung Institute
Building 31, Room 4A-11
Bethesda, Maryland 20014

Technical
Dr. Suzanne S. Hurd

Assigned to: Chemistry

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RA-3 (6-71)

GEORGIA INSTITUTE OF TECHNOLOGY
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SPONSORED PROJECT TERMINATION

Posted 4/15 OHL

Date: April 9, 1976

Project Title: "Synthesis of Elastase Inhibitors"

Project No: G-33-675

Project Director: Dr. James C. Powers

Sponsor: Public Health Service, NIH

Effective Termination Date: August 31, 1975 (Contract Expiration)

Clearance of Accounting Charges: N/A - Final Billing in Process.

~~Grant~~/Contract Closeout Actions Remaining: None.

Property Certificate submitted 26 Jan 76

- _ Final Invoice and Closing Documents
- _ Final Fiscal Report
- _ Final Report of Inventions
- _ Govt. Property Inventory & Related Certificate
- _ Classified Material Certificate
- _ Other _____

Assigned to: Chemistry (School/Laboratory)

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G-33-615

BRIEF PROGRESS REPORT

SYNTHESIS OF ELASTASE INHIBITORS

NIH CONTRACT NO. NO1 HR 42939

Submitted by

James C. Powers
Principal Investigator
Department of Chemistry
Georgia Institute of Technology
Atlanta, Georgia 30332

August 31, 1974

Synthesis of the Chloroketones Ac-Ala-Ala-Ala-AlaCH_2Cl and Ac-Ala-Ala-Pro-AlaCH_2Cl. As of Aug 31, 1974, the synthesis of Ac-Ala-Ala-Pro-AlaCH_2Cl has been carried out once on a small scale (yield 200mg) and once on a large scale (yield 4-6g) by James Boone (a postdoctorial fellow who started the synthesis on June 15, 1974). The product from the large scale reaction has not yet been purified and crystalized. Many of the synthetic intermediates have been prepared in large amounts and in particular Ac-Ala-Ala-OH and Z-AlaCH_2Cl will be used in the synthesis of the second chloroketone, Ac-Ala-Ala-Ala-AlaCH_2Cl.

Distribution of Inhibitors. A summary of the requests for inhibitors prepared by Dr. Clark Lum (letter of Aug. 19, 1974) indicates an initial need for 0.5g of each chloromethyl ketone inhibitor. We have on hand 0.5g of Ac-Ala-Ala-Ala-AlaCH_2Cl from an earlier synthesis. We expect to have Ac-Ala-Ala-Pro-AlaCH_2CL purified and crystalized next week. We will therefore begin to mail out the inhibitors next week. I will contact Dr. Clark by phone to discuss details of the shipping.

Study of the Inhibition of Leukocyte Elastase. A study of the rate of reaction of human leukocyte elastase (1.6mg kindly provided to us by Dr. Aaron Janoff at the State University of New York, Stony Brook) at pH 6.5 with 10 chloromethyl ketones has been completed (a preprint of a manuscript detailing our results is attached). The most effective inhibitor was Ac-Ala-Ala-Pro-AlaCH_2Cl(relative rate 15.8 compared to that of Ac-Ala-Ala-AlaCH_2Cl which was 1.0). Other relative inhibitors were Z-Gly-Leu-AlaCH_2Cl (7.3) and Ac-Ala-Pro-Ala-AlaCH_2Cl (8.1). Ac-Ala-Ala-Ala-AlaCH_2Cl had a relative rate of 3.5.

Synthesis of Other Elastase Inhibitors. Mr. David Carroll has recently synthesized the carbozate Z-Ala-Ala-Pro-NH-N(CH$_3$)-CO-ONp

This irreversibly acylated porcine elastase. Preliminary experiments with human leukocyte elastase (before we ran out of enzyme) indicate that this compound is also an effective inhibitor of the human enzyme. We are pursuing synthesis and enzymatic studies with this new class of elastase inhibitors.

SYNTHETIC ACTIVE SITE-DIRECTED INHIBITORS OF ELASTOLYTIC PROTEASES *

James C. Powers, David L. Carroll, and Peter M. Tuhy †

School of Chemistry
Georgia Institute of Technology
Atlanta, Georgia 30332

Several proteolytic enzymes are known to occur in human polymorphonuclear (PMN) leukocyte granule fractions, including an elastase that is active at physiologic pH.[1-4] The human leukocyte elastase has been isolated and purified by affinity chromatography. It is known to degrade human lung elastin, arterial walls, and basement membrane, digest proteins of bacterial cell walls *in vitro*, induce cellular surface changes correlated with loss of growth control, and undergo inhibition by α_1-antitrypsin and peptide chloromethyl ketones.[5-9] A growing body of experimental evidence has suggested that this elastase may be involved in pathologic processes associated with elastic tissue damage in acute arthritis, pulmonary emphysema, and related diseases.[10-13] Thus, synthetic elastase inhibitors would be expected to be useful reagents both for the treatment of arthritis, emphysema, and related diseases and for the study of the biologic function of elastolytic enzymes.

Human leukocyte elastase is similar to the more widely studied porcine pancreatic elastase [14] in many respects. They are both serine proteases and show esterase activity toward synthetic substrates, such as Boc-Ala-ONp and Ac-Ala-Ala-Ala-OCH_3.

One very useful class of inhibitors for serine proteases is the chloromethyl ketones. These inhibitors, if properly designed to resemble a substrate, are fairly specific for one serine protease or a group of serine proteases with similar specificities.[15] The reason for this specificity is apparent upon consideration of the mechanism of action of serine proteases. A substrate is bound in such a way that the carbonyl group of the scissile peptide bond is properly oriented to be attacked by the active-site serine residue, which action leads to an acyl enzyme intermediate that rapidly turns over (FIGURE 1). A similarly bound chloromethyl ketone inhibitor (FIGURE 1), however, would place the active-site histidine residue next to the alkylating group of the inhibitor, which results in the formation of a covalent bond between enzyme and inhibitor. Attack by the active-site serine residue on the carbonyl group of the inhibitor would lead to no stable products.

A series of alanine tri- and tetrapeptide chloromethyl ketones was recently synthesized by us [16, 17] and subsequently by two independent groups [18, 19] as elastase inhibitors. The inhibitors were designed to contain two features thought to be essential for effective inhibition, a terminal alanine chloromethyl ketone moiety due to the known specificity of elastase and an extended peptide chain, because the rate of elastase hydrolysis of synthetic substrates is strongly

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dependent on peptide chain length. Most of the compounds synthesized by us were effective inhibitors of porcine pancreatic elastase; Ac-Ala-Ala-Pro-$AlaCH_2Cl$ was the most reactive. The tetrapeptide inhibitors were 10–50 times more reactive than the tripeptides. The related serine proteases chymotrypsin and trypsin were not significantly inhibited by these peptide chloromethyl ketones under identical reaction conditions. Several of our compounds, including Ac-Ala-Ala-Ala-$AlaCH_2Cl$ and Ac-Ala-Ala-Pro-$AlaCH_2Cl$, were also shown by Janoff [10] to be effective inhibitors of the human leukocyte elastase.

We have recently completed a kinetic study of the inhibition of human leukocyte elastase by a series of peptide chloromethyl ketones that demonstrates that the human leukocyte enzyme is distinct from the porcine pancreatic enzyme. Human leukocyte elastase, 85% pure as determined by a gel densitometry scan, which contains one major and two minor elastase isoenzymes, was kindly provided by Dr. A. Janoff. Inhibition reactions were performed by

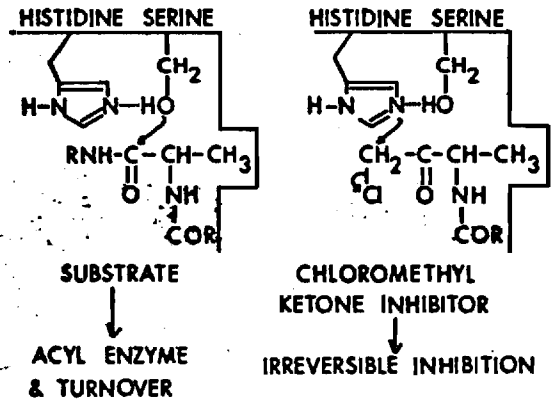

FIGURE 1. Comparison of the reaction pathways for the reaction of elastase with a substrate and with a chloromethyl ketone.

rapidly mixing elastase and inhibitor solutions (0.1 M phosphate, pH 6.5) at 25° C and allowing the reaction to proceed through at least two half-lives. Initial concentrations were: elastase, 10μM; tripeptide inhibitor, 1 mM (or tetrapeptide inhibitor, 0.2 mM); $CaCl_2$, 0.1 mM; methanol, 5% v/v. Enzyme assays were periodically performed with the Boc-Ala-ONp spectrophotometric assay at 345 nm.[20] Kinetic results for each inhibitor were calculated from the equation

$$v_{\text{inhib}} = k_{\text{obsd}}[E] = k_{\text{2nd}}[I][E]$$

with the aid of a line-fitting computer program and are presented in TABLE 1. The second-order rate constant $k_{\text{obsd}}/[I]$ is suitable only for purposes of approximate reactivity comparisons among the inhibitors, because $k_{\text{obsd}}/[I]$ becomes nonlinear with $[I]$ when the latter approaches K_I, the dissociation constant of the enzyme-inhibitor complex.[16, 17] Slight problems of reproducibility arose from a lack of enough enzyme for duplicating all inhibition runs, but the correlation coefficients for all runs were greater than 0.99.

TABLE 1

INHIBITION OF HUMAN LEUKOCYTE ELASTASE WITH PEPTIDE CHLOROMETHYL KETONES (ELASTASE CONCENTRATION 10 μM, AT PH 6.5, 25° C, 5% METHANOL)

Inhibitor P_4 P_3 P_2 P_1	$10^4 \cdot k_{obsd}$ (sec^{-1})	$10 \cdot k_{obsd}/[I]$ ($M^{-1} sec^{-1}$)	$k_{obsd}/[I]$ (rel.)	$k_{obsd}/[I]$ * (rel.)
Ac-Ala-Ala-AlaCH$_2$Cl	2.8 †	2.8	1.0	9.3
Ac-Ala-Pro-AlaCH$_2$Cl	10.9	10.9	4.0	14.9
Ac-Pro-Ala-AlaCH$_2$Cl	0	0	0	0
Z-Gly-Leu-AlaCH$_2$Cl	20.2	20.2	7.3	17.6
Z-Gly-Leu-PheCH$_2$Cl	0	0	0	—
Ac-Ala-Ala-Ala-AlaCH$_2$Cl	2.0 †	9.7	3.5	167
Ac-Ala-Ala-Pro-AlaCH$_2$Cl	8.7 †	43.6	15.8	650
Ac-Ala-Pro-Ala-AlaCH$_2$Cl	4.4	22.2	8.1	0
Ac-Ala-Ala-Phe-AlaCH$_2$Cl	2.6	13.2	4.8	149
Ac-Phe-Gly-Ala-LeuCH$_2$Cl	1.2	6.0	2.2	

* Data for porcine pancreatic elastase.[6]
† Average of two runs.

A comparison of the effectiveness of the peptide chloromethyl ketone inhibitors, based on their relative $k_{obsd}/[I]$ values, leads to several interesting relationships between the structure of inhibitors and reactivity toward human leukocyte elastase. The tetrapeptides were usually observed to be faster inhibitors than the tripeptides and were run at fivefold lower inhibitor concentrations, [*I*]. Ac-Ala-Ala-Pro-AlaCH$_2$Cl is the best leukocyte elastase inhibitor of the series. In general, the tetrapeptides are about three to four times more reactive than the tripeptides, which is evidence for the inhibitors' interaction with an extended binding site in leukocyte elastase. The P_1 residue[21] is alanine in almost all of the inhibitors, for example, Ac-Ala-Ala-Ala-*Ala*CH$_2$Cl, which corresponds to the substrate specificity of elastase for alanine and other amino acids with small side chains. If P_1 is phenylalanine, as in the chymotrypsin inhibitor Z-Gly-Leu-*Phe*CH$_2$Cl, no leukocyte elastase inhibition is observed, whereas if P_1 is leucine, as in the potent subtilisin inhibitor Ac-Phe-Gly-Ala-*Leu*CH$_2$Cl, a small rate of elastase inhibition occurs. In the P_2 position, proline appears to be favored over alanine: $k_{obsd}/[I]$ for Ac-Ala-Ala-*Pro*-AlaCH$_2$Cl is four to five times larger than that for Ac-Ala-Ala-*Ala*-AlaCH$_2$Cl, and this also holds true for the analogous tripeptide pair. A leucine in P_2 is also favorable, as in Z-Gly-*Leu*-AlaCH$_2$Cl. If the P_3 residue is proline, as in the tripeptide inhibitor Ac-*Pro*-Ala-AlaCH$_2$Cl, leukocyte elastase inhibition is totally blocked, but if P_3 is proline, as in the tetrapeptide inhibitor Ac-Ala-*Pro*-Ala-AlaCH$_2$Cl, elastase inhibition does occur. Ac-Ala-*Pro*-Ala-AlaCH$_2$Cl is a noninhibitor toward porcine pancreatic elastase.[16, 17] For optimum reactivity, a leukocyte elastase inhibitor should contain a P_1 alanine, a P_2 proline or leucine, and no P_3 proline (for tripeptides).

At this point, it is of interest to examine certain inhibition characteristics of human leukocyte elastase, as compared to the more thoroughly studied porcine pancreatic elastase.[16, 17] First, leukocyte elastase reacts less rapidly overall with this group of peptide chloromethyl ketones than does pancreatic elastase, as indicated by smaller values of $k_{obsd}/[I]$ for inhibition (TABLE 1). Second, the increase in reactivity toward inhibitors in going from tripeptides to

tetrapeptides is smaller for leukocyte elastase, which fact may indicate differences in the geometry of the extended binding sites and the effect of the individual subsites on the catalytic activity of these two enzymes. Third, the derived structure-reactivity relationships are generally parallel between the inhibitors and the two kinds of elastase, with regard to the structural features required for enhancement of elastase inhibition. Finally, Ac-Ala-Ala-Pro-AlaCH_2Cl is the most effective inhibitor tested for both human leukocyte and porcine pancreatic elastase. Interestingly, its isomer Ac-Ala-Pro-Ala-AlaCH_2Cl is a relatively good inhibitor for the leukocyte enzyme but is unable even to touch the pancreatic enzyme, which is strong evidence that the leukocyte and pancreatic enzymes are distinct entities.

Another useful class of inhibitors for serine proteases is the peptide carbazates. Carbazates with the appropriate substituents are analogs of amino acids in which the α-methine group has been replaced by a nitrogen atom. They would thus be expected to acylate elastase in much the same fashion as simple synthetic peptide substrates. The carbazoyl enzyme should be considerably more stable toward deacylation than is a normal acyl enzyme due to the influence of the adjacent nitrogen atom (FIGURE 2).

The reaction of serine proteases with two carbazates has been previously studied. Ethyl 3-acetyl-2-benzylcarbazate is not hydrolyzed by chymotrypsin but is, however, a predominantly competitive inhibitor of the enzyme.[22] On the other hand, 4-nitrophenyl 3-acetyl-2-benzylcarbazate acylates chymotrypsin and trypsin and has been used as an active-site titrant.[23]

We have synthesized two peptide carbazates as potential inhibitors of elastase, Ac-Ala-Mec-ONp,*1* (Mec = 3-methyl carbazoic acid) with Z-Ala-Ala-Pro-Mec-ONp,*2* (FIGURE 3). Dipeptide *1* does not react with either pancreatic elastases or with human leukocyte elastase, an observation that is not surprising in view of the requirement of elastase for an extended peptide chain in a substrate [24] or inhibitor.[16, 17] Tetrapeptide *2* reacted with porcine pancreatic elastase (inhibitor to enzyme ratio = 47) at pH 5.0 (0.1 M acetate) or pH 5.8 (0.1 M citrate) to give a burst of 4-nitrophenol that was complete in less than 9 sec. Presumably, a carbazoyl enzyme is formed as the other product. The

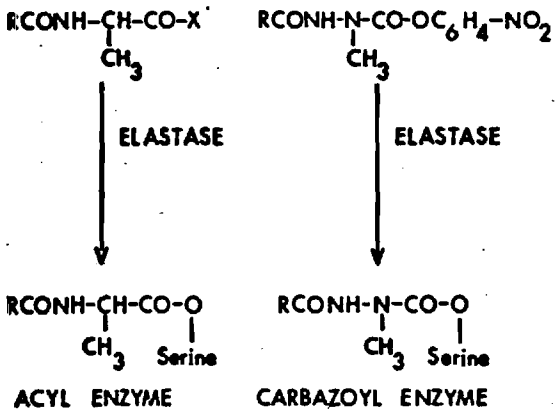

FIGURE 2. Comparison of the structures of an acyl enzyme and a carbazoyl enzyme formed from elastase.

inactive enzyme thus formed, however, regains activity gradually as the carbazoyl group is hydrolyzed slowly from the enzyme ($t_{1/2}$ at pH 5.0 = 6.2×10^2 sec). Human leukocyte elastase (inhibitor to enzyme ratio = 10^3) is also rapidly inactivated by Z-Ala-Ala-Pro-Mec-ONp (*2*) at pH 6.5 (0.1 M phosphate). In this case, the reactivation is fairly slow, but a lack of sufficient enzyme precluded a careful study of the deacylation reaction. Unfortunately, Z-Ala-Ala-Pro-Mec-ONp (*2*) was not specific for elastase but also acylated subtilisin BPN′ and chymotrypsin Aγ.

Although Z-Ala-Ala-Pro-Mec-ONp is not a useful inhibitor for elastase, it can be used as an active-site titrant. The release of 4-nitrophenol is stoichiometric, and the deacylation rate, although not negligible, is much slower than the initial acylation. The reagent could not be used, however, in mixtures that contained chymotrypsin-like enzymes due to the nonspecificity for the reaction of this particular carbazate. We expect to be able to increase the specificity of carbazates for elastase by alteration of the extended peptide structure of the reagent. In addition, we feel that it is also possible to increase the stability of the carbazoyl enzyme in a like manner, which would thus make the parent carbazates effective inhibitors. This goal has already been realized for a

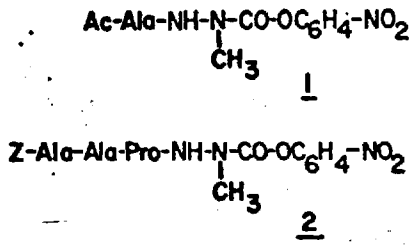

FIGURE 3. Peptide carbazates synthesized as elastase inhibitors.

carbazoyl derivative of chymotrypsin that has a half-life for deacylation of several days.

In conclusion, we have clearly demonstrated that peptide chloromethyl ketones are valuable active-site-directed inhibitors of human leukocytic and porcine pancreatic elastase and that peptide carbazates are useful active-site titrants for elastase and are potentially effective inhibitors. At present, several investigators have initiated *in vivo* studies with chloromethyl ketone inhibitors, and they are proving useful for elucidating many of the molecular pathways by which human leukocyte elastase performs its physiologic and pathologic functions. In the future, these or related inhibitors should prove to be useful for the treatment of arthritis, pulmonary emphysema, and other diseases that involve elastic tissue destruction by elastolytic proteases.

ACKNOWLEDGMENT

We thank Dr. Aaron Janoff at the State University of New York at Stony Brook for kindly providing the 1.6 mg of human leukocyte elastase used in this work.

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INHIBITION OF HUMAN LEUKOCYTE ELASTASE BY PEPTIDE CHLOROMETHYL KETONES

Peter M. TUHY and James C. POWERS*
School of Chemistry, Georgia Institute of Technology, Atlanta, Georgia 30332 USA

Received 2 December 1974

1. Introduction

Recently, an elastolytic protease has been isolated from human polymorphonuclear (PMN) leukocyte granule fractions and purified by affinity chromatography [1]. This human leukocyte elastase is known to degrade human lung elastin, arterial walls and basement membrane, digest proteins of bacterial cell walls in vitro, induce cellular surface changes correlated with loss of growth control, and undergo inhibition by α_1-antitrypsin and peptide chloromethyl ketones [2]. A growing body of experimental evidence has suggested that this elastase may be involved in pathological processes associated with elastic tissue damage in pulmonary emphysema and acute arteritis, and with the tumor-producing action of cocarcinogenic substances [3]. Peptide chloromethyl ketones are well-known inhibitors of serine proteases which have proved quite useful for structural studies, including X-ray crystallographic investigations, of the active site regions of this group of enzymes [4]. A series of alanine tri- and tetrapeptide chloromethyl ketone inhibitors was synthesized by us and two other groups to probe the nature of the extended binding site and catalytic activity of porcine pancreatic elastase (EC 3.4.21.11) and related enzymes [5,6]. In this paper we report a study of the inhibition of human leukocyte elastase by a series of peptide chloromethyl ketones, which demonstrates that the human leukocyte enzyme is distinct from the porcine pancreatic enzyme. The results also define the structural features necessary for an effective inhibitor and may lead to a treatment for emphysema and related diseases.

* To whom requests for reprints should be sent.

2. Materials and methods

Human leukocyte elastase was 85% pure as determined by a gel densitometry scan and contained one major and two minor elastase isoenzymes. Inhibition reactions were performed by rapidly mixing elastase and inhibitor solutions in 0.1 M phosphate, pH 6.5, at 25°C, and allowing the reaction to proceed through at least two half-lives. Final concentrations were: elastase, 10 μM; tripeptide inhibitor, 1 mM (or tetrapeptide inhibitor, 0.2 mM); $CaCl_2$, 0.1 mM; methanol, 5% v/v; in a total volume of 0.31 ml. These conditions were chosen to minimize the enzyme consumed and to facilitate kinetic measurements. Enzyme assays were periodically performed on aliquots containing 12.5 μg elastase using the BOC-Ala-ONp spectrophotometric assay method at 345 nm [7]. Kinetic results for each inhibitor were calculated from the equation

$$V_{\text{inhibition}} = k_{\text{obsd}}(\text{E}) = k_{\text{2nd}}\,(\text{I})\,(\text{E})$$

with the aid of a line-fitting computer program, and are presented in table 1. The second-order rate constant $k_{\text{obsd}}/(\text{I})$ is suitable only for purpose of approximate reactivity comparisons among the inhibitors, since $k_{\text{obsd}}/(\text{I})$ becomes non-linear with (I) when (I) becomes close to K_I, the dissociation constant of the enzyme–inhibitor complex [5]. Due to the small amount of enzyme available we were unable to carry out concentration-dependent inhibition studies to determine K_I values. Slight problems of reproducibility arose from a lack of enough enzyme for duplicating all inhibition runs, but the correlation coefficients for all runs were greater than 0.99.

Table 1
Inhibition of human leukocyte elastase with peptide chloromethyl ketones

Inhibitor P_4 P_3 P_2 P_1	(I) (mM)	$10^4 \cdot k_{obsd}$ (sec^{-1})	$10 \cdot k_{obsd}/(I)$ ($M^{-1} sec^{-1}$)	$k_{obsd}/(I)$ (rel.)	$k_{obsd}/(I)$* (rel.)
Ac–Ala–Ala–$AlaCH_2Cl$	1.0	2.8**	2.8	1.0	1.0
Ac–Ala–Pro–$AlaCH_2Cl$	1.0	10.9	10.9	4.0	1.6
Ac–Pro–Ala–$AlaCH_2Cl$	1.0	0	0	0	0
Z–Gly–Leu–$AlaCH_2Cl$	1.0	20.2	20.2	7.3	1.9
Z–Gly–Leu–$PheCH_2Cl$	1.0	0	0	0	–
Ac–Ala–Ala–Ala–$AlaCH_2Cl$	0.2	2.0**	9.7	3.5	18
Ac–Ala–Ala–Pro–$AlaCH_2Cl$	0.2	8.7**	43.6	15.8	71
Ac–Ala–Pro–Ala–$AlaCH_2Cl$	0.2	4.4	22.2	8.1	0
Ac–Ala–Ala–Phe–$AlaCH_2Cl$	0.2	2.6	13.2	4.8	16
Ac–Phe–Gly–Ala–$LeuCH_2Cl$	0.2	1.2	6.0	2.2	–

* Data for porcine pancreatic elastase [5].
** Average of two runs.
Elastase concentration 10 μM, at pH 6.5, 25°C, 5% methanol.

3. Results and discussion

A comparison of the effectiveness of the peptide chloromethyl ketone inhibitors, based on their relative $k_{obsd}/(I)$ values, leads to several interesting relationships between the structure of inhibitors and reactivity toward human leukocyte elastase. The tetrapeptides were usually observed to be faster inhibitors than the tripeptides and were run at 5-fold lower inhibitor concentrations (I). Ac–Ala–Ala–Pro–$AlaCH_2Cl$ is the best leukocyte elastase inhibitor of the series. In general the tetrapeptides are about 3 to 4 times more reactive than the tripeptides, which is evidence for the inhibitors' interaction with an extended binding site in leukocyte elastase. The P_1 residue [8] is alanine in almost all of the inhibitors, e.g. Ac–Ala–Ala–Ala–*Ala*CH_2Cl, corresponding to the substrate specificity of elastase for alanine and other amino acids with small side chains. If P_1 is phenylalanine, as in the chymotrypsin inhibitor Z–Gly–Leu–*Phe*CH_2Cl, no leukocyte elastase inhibition is observed, whereas if P_1 is leucine, as in the potent subtilisin inhibitor Ac–Phe–Gly–Ala–*Leu*CH_2Cl, a small rate of elastase inhibition takes place. In the P_2 position, proline appears to be favored over alanine: $k_{obsd}/(I)$ for Ac–Ala–Ala–*Pro*–$AlaCH_2Cl$ is 4 to 5 times larger than for Ac–Ala–Ala–*Ala*–CH_2Cl, and this also holds true for the analogous tripeptide pair. A leucine in P_2 is also favorable as in Z–Gly–*Leu*–$AlaCH_2Cl$, but a phenylalanine in P_2 is little better than alanine: compare Ac–Ala–Ala–*Phe*–$AlaCH_2Cl$ with Ac–Ala–Ala–*Ala*–$AlaCH_2Cl$. Thus human leukocyte elastase may possibly exhibit a weak secondary specificity for inhibitors with P_2 residues possessing medium-sized alkyl side chains (4). If the P_3 residue is proline as in the tripeptide inhibitor Ac–*Pro*–Ala–Ala–CH_2Cl, no inhibition of leukocyte elastase is observed. But if P_3 is proline as in the tetrapeptide inhibitor Ac–Ala–*Pro*–Ala–Ala–CH_2Cl, elastase inhibition does occur. Ac–Ala–*Pro*–Ala–Ala–CH_2Cl is a non-inhibitor toward porcine pancreatic elastase [5]. In the P_4 position a large hydrophobic group, e.g. phenyl, appears to be beneficial although further studies will be necessary for verification. This could explain the higher than expected activity of Z–Gly–Leu–$AlaCH_2Cl$ and Ac–*Phe*–Gly–Ala–$LeuCH_2Cl$, both of which possess a benzyl side chain at P_4, toward leukocyte elastase. For optimum reactivity, a leukocyte elastase inhibitor should contain a P_1 alanine, a P_2 proline or leucine, no P_3 proline (in the case of tripeptides), and possibly a P_4 phenylalanine.

At this point, it is of interest to examine certain inhibition characteristics of human leukocyte elastase as compared to the more thoroughly studied porcine pancreatic elastase [5]. First of all, leukocyte elastase reacts less rapidly overall with this group of peptide chloromethyl ketones than does pancreatic elastase, as indicated by smaller values of $k_{obsd}/(I)$ for inhibition. Secondly, the increase in reactivity toward inhi-

bitors in going from tripeptides to tetrapeptides is smaller in the case of leukocyte elastase, which may indicate differences in the geometry of the extended binding sites and the effect of the individual subsites on the catalytic activity of these two enzymes. Thirdly, the derived structure–reactivity relationships are generally parallel between the inhibitors and the two kinds of elastase, with regard to the structural features required for enhancement of elastase inhibition. Finally, Ac–Ala–Ala–Pro–AlaCH_2Cl is the most effective inhibitor tested for both human leukocyte and porcine pancreatic elastase. Interestingly, its isomer Ac–Ala–Pro–Ala–AlaCH_2Cl is a relatively good inhibitor for the leukocyte enzyme but is not effective to touch the pancreatic enzyme, which is strong evidence that the leukocyte and pancreatic enzymes are distinct entities.

In conclusion, this initial kinetic study of human leukocyte elastase clearly demonstrates the value of peptide chloromethyl ketones as active-site directed inhibitors for this enzyme in providing structure–reactivity correlations. At present a number of investigators have begun in vivo enzymatic studies with these inhibitors and they should prove useful for elucidating many of the molecular pathways by which human leukocyte elastase carries out its physiological and pathological functions.

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We wish to thank Dr Aaron Janoff at the State University of New York at Stony Brook for kindly providing the 1.6 mg of human leukocyte elastase used in this work. This research was supported by a Research Corporation grant and NIH Contract HR-42939, and a NDEA Traineeship for 1972-1974 is gratefully acknowledged (P.T.).

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SYNTHESIS OF ELASTASE INHIBITORS

James C. Powers, Ph.D.
School of Chemistry
Georgia Institute of Technology
Atlanta, Georgia 30332

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Division of Lung Diseases
National Heart and Lung Institute
National Institute of Health
Bethesda, Maryland 20014

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16. Abstracts

Pulmonary emphysema is currently thought to result from the uninhibited proteolysis of lung tissue by elastase and other proteases. Synthetic inhibitors capable of specifically inhibiting elastase would be expected to be useful reagents both for the treatment of emphysema and for the study of the biological function of elastolytic enzymes.

Human leukocyte elastase is irreversibly inhibited by peptide chloromethyl ketones. The peptide chloromethyl ketones Ac-Ala-Ala-Ala-AlaCH_2Cl and Ac-Ala-Ala-Pro-AlaCH_2Cl were synthesized and distributed to over 30 other investigators for use in the study of this enzyme and its role in lung disease. Studies of the rate of inhibition of human leukocyte elastase by chloromethyl ketones led to the design and synthesis of Ac-Ala-Ala-Pro-ValCH_2Cl and Ac-Ala-Ala-Pro-IleCH_2Cl which are 49 and 40 fold respectively more effective inhibitors than Ac-Ala-Ala-Pro-AlaCH_2Cl. A number of papers have already been published in which these peptide chloromethyl ketones have been utilized.

Peptide carbazates have been studied as possible elastase inhibitors. Ac-Ala-Ala-Mec-ONp (Mec=NHN(CH_3)CO-) acylates elastase within a few seconds with concurrent inhibition of the enzyme. However, the acylation is reversible and the enzyme regains activity in 0.25 to 1 hr. The carbazate is a useful reagent for titrating elastase and has been distributed to several other investigators.

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TABLE OF CONTENTS

		Page
I.	Summary	3
II.	Introduction	3-4
III.	Goals	5
IV.	Background	
	Elastase	5-8
	Elastase Inhibitors	8-9
V.	Peptide Chloromethyl Ketones	
	Rationale	9-12
	Synthetic Results	12
	Distribution	12-14
	Inhibition of Human Leukocyte Elastase	14-15
	Published Studies of Other Investigators	15-20
VI.	Peptide Carbazates	20
	Synthetic Results and Distribution	20-23
	Inhibition of Human Leukocyte Elastase	23
VII.	Results and Conclusions	23-24
VIII.	References	25-26
IX.	Personnel Associated with Project	27
X.	Publications	
	Appendix	

I. Summary

Pulmonary emphysema is currently thought to result from the uninhibited proteolysis of lung tissue by elastase and other proteases. Synthetic inhibitors capable of specifically inhibiting elastase would be expected to be useful reagents both for the treatment of emphysema and for the study of the biological function of elastolytic enzymes.

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Peptide carbazates have been studied as possible elastase inhibitors. Ac-Ala-Ala-Mec-ONp (Mec = $-NHN(CH_3)CO-$) acylates elastase within a few seconds with concurrent inhibition of the enzyme. However, the acylation is reversible and the enzyme regains activity in 0.25 to 1 hr. The carbazate is a useful reagent for titrating elastase and has been distributed to several other investigators.

II. Introduction

Pulmonary emphysema is currently thought to result from the uninhibited proteolysis of lung tissue by elastase and related neutral proteases derived from leukocytes, alveolar macrophages or other cellular sources (Mittman, 1972). During infection or inflammation, the

normal lung is protected from proteolytic digestion by the serum protease inhibitor, α_1-antitrypsin. Individuals with an inherited deficiency of α_1-antitrypsin often develop emphysema at an early age. In addition to α_1-antripsin deficiency, emphysema is also probably related to the rate of release of proteases in lung tissue either from exogenous sources (e.g. bacterial infection) or by the stimulation of normal cellular protease producing pathways by environmental factors (smoking, air pollution, etc.). Thus, individuals with normal α_1-antitrypsin levels, but with high rates of protease production are also susceptible to lung disease.

Synthetic inhibitors capable of specifically inhibiting elastase would be expected to be useful reagents both for the treatment of emphysema and related diseases and for the study of the biological function of elastolytic enzymes. Such compounds could potentially be utilized to replace α_1-antitrypsin in individuals with an inherited deficiency. They might also be useful for the protection of individuals with high rates of protease release into lung tissue. Since emphysema is characterized by the gradual evolution of lesions in the lung due to proteolytic destruction of elastin in the alveolar structure, elastase inhibitors should also be capable of arresting the further development of the disease in individuals already inflicted with emphysema.

This contract dealt with the synthesis of peptide chloromethyl ketones which we have shown to be highly reactive and specific elastase inhibitors (Powers and Tuhy, 1972 and 1973; Tuhy and Powers, 1975) and with the design and synthesis of other elastase inhibitors with a variety of functional groups. Many of our elastase inhibitors have already been used or are currently being used by other investigators engaged in the study of the role of proteolytic enzymes in pathological lung disease.

III. Goals

1. Synthesize 10-50 grams of the chloromethyl ketones Ac-Ala-Ala-Ala-AlaCH$_2$Cl and Ac-Ala-Ala-Pro-AlaCH$_2$Cl.

2. Study the inhibition of leukocyte elastase with a series of peptide chloromethyl ketones in order to optimize the inhibitor structure.

3. Synthesize other elastase inhibitors such as peptide carbazates with more desirable properties such as solubility, hydrophobicity, reactivity and specificity.

4. Distribute inhibitors including carbazates to investigators who have submitted justified requests to the staff of the Division of Lung Diseases.

IV. Background

Elastase. Elastase is a serine protease homologous to chymotrypsin and trypsin which digests elastin, the flexible protein component of lung and elastic connective tissue. Elastase activity has been found in a variety of locations, including human pancreatic extracts (Hartley and Shotton, 1971), human peripheral blood polymorphonuclear leukocytes and alveolar macrophages (Janoff, 1972).

Pancreatic porcine elastase has been extensively studied due to its ready availability. Studies of its substrate specificity using N-benzyloxycarbonylamino acid p-nitrophenyl esters (Geneste and Bender, 1969), N-benzoylamino acid methyl esters (Kaplan, et. al., 1970), oxidized insulin A and B chains (Narayanian and Awar, 1969), and ribonuclease S peptide (Atlas, et. al., 1970) have shown that porcine pancreatic elastase cleaves most readily peptide bonds C terminal to alanine residues. This is consistent with the observation that elastin, elastase's normal substrate, contains a high percentage of alanine, glycine and proline. More recent work with synthetic peptides has shown that the rate of elastase hydrolysis is strongly dependent on

chain length (Atlas, et. al., 1970; Atlas and Berger, 1972; Thompson and Blout, 1970, 1973b) with a substantial increase occuring on going from tripeptide to tetrapeptide substrates (Thompson and Blout, 1973b). These results indicate that elastase, like the serine proteases chymotrypsin (Segal, et. al., 1971 a and b) and subtilisin (Kraut, et. al., 1971; Robertus, et. al., 1972), contains an extended substrate binding site.

The three dimensional structure of porcine pancreatic elastase has been determined by X-ray crystallography (Hartley and Shotton, 1971; Shotton, et. al., 1972). The catalytic site consists of a charge relay system (Figure 1) composed of an aspartic acid residue, a serine residue (Ser-188) and the imidazole ring of a histidine residue (His-45). During elastase catalyzed hydrolysis of a peptide substrate, Ser-188 is acylated by the acyl portion of the peptide bond that is cleaved (see Figure 2). A shallow pocket is located on the surface of the enzyme adjacent to the catalytic residues. This region of the enzyme is responsible for the observed specificity of elastase since only the side chains of certain amino acid residues (e.g., alanine) can fit into this pocket. Crystallographic experiments to elucidate the nature of the extended substrate binding region of porcine pancreatic elastase are in progress (D. M. Shotton, personal communication), although I expect it to be very similar to that observed in the homologous serine proteases, chymotrypsin and subtilisin.

Knowledge of the leukocyte elastase(s) is much more limited. However, the few experiments that have been carried out with the leukocyte elastase have left little doubt concerning the essential similarity of the pancreatic enzyme and the leukocyte elastase. For example, the leukocyte enzyme can be assayed using the same synthetic substrates Boc-Ala-ONp and Ac-Ala-Ala-Ala-OMe (Janoff, 1969; Janoff and Basch, 1971) that were

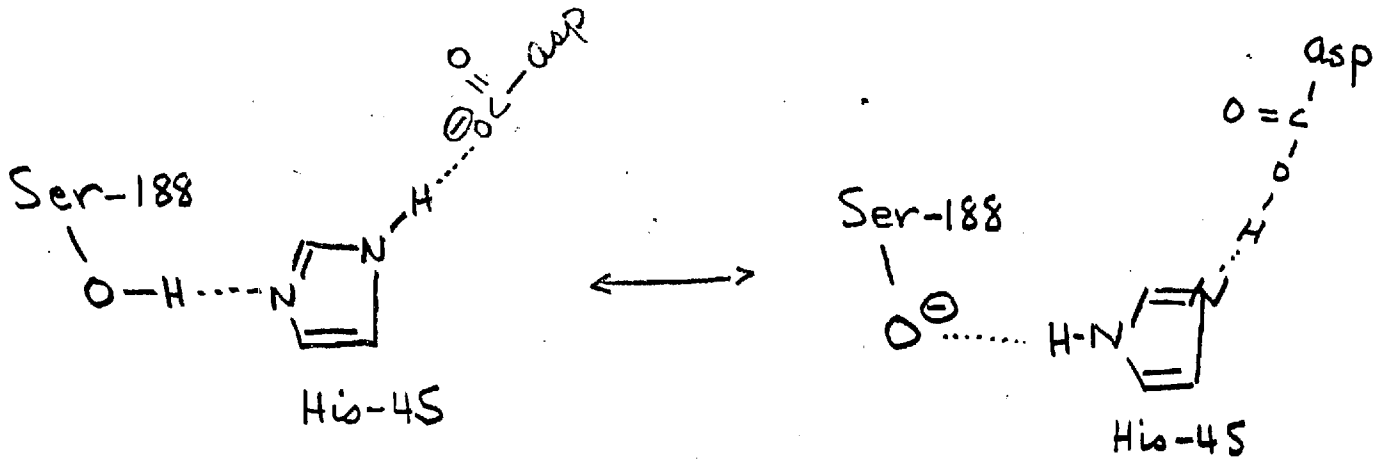
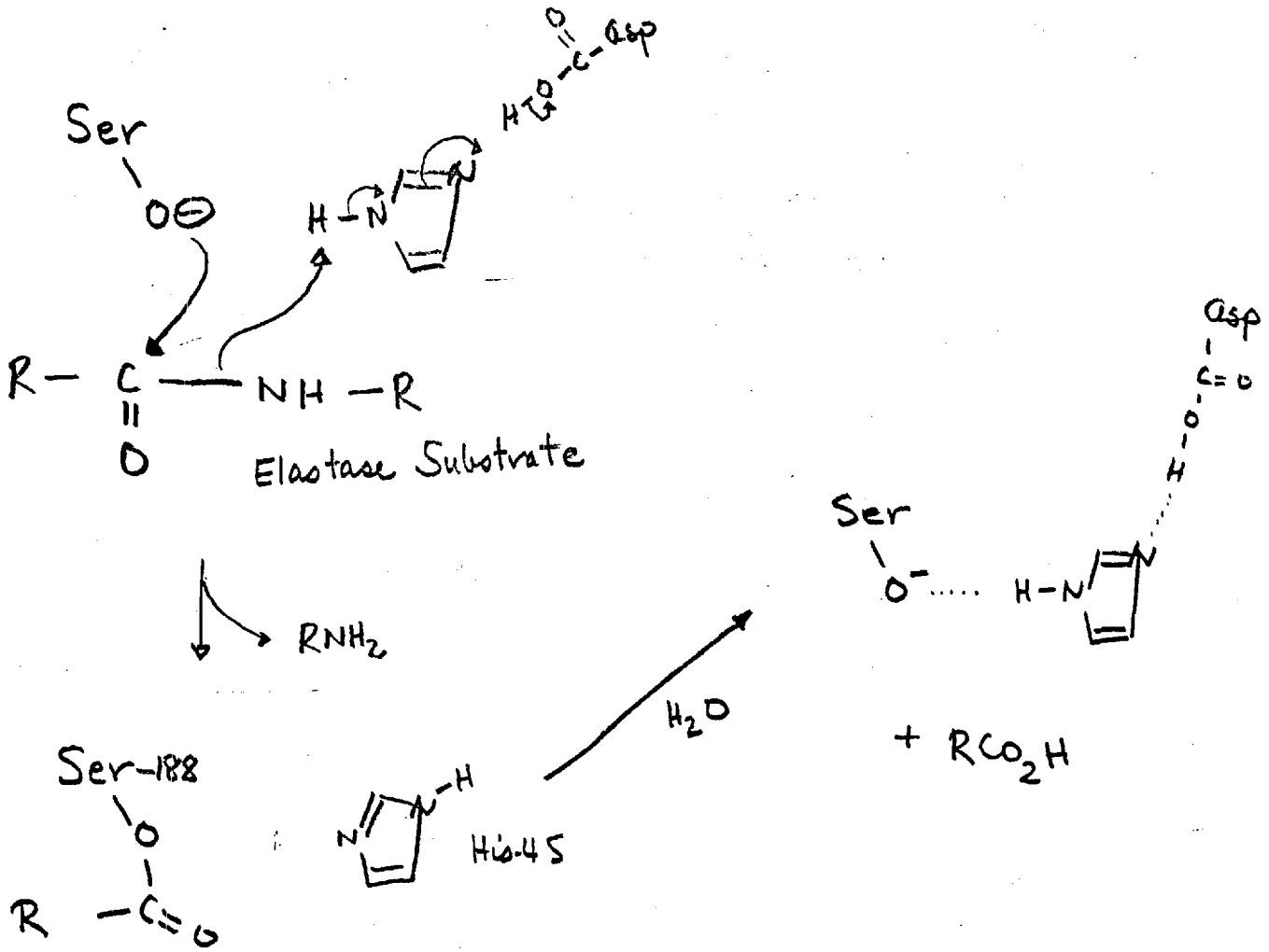


Fig. 1 - Active site of Elastase (Two resonance forms)



Elastase Acylated by Half of the Substrate

Fig. 2 - Outline of Elastase's Hydrolysis Mechanism

developed to assay porcine pancreatic elastase. The pH optimiums of the two enzymes are similar (Janoff and Scherer, 1968). The human granulocyte elastase is inhibited by diisopropylphosphoro fluoridate, a reaction characteristic of serine proteases, and by serum α_1-antitrypsin (Janoff, 1972 a). The two enzymes differ in certain respects. The pancreatic enzyme is inhibited by soybean trypsin inhibitor, salivary kallikrein inhibitor, and by high salt concentrations while the leukocyte enzyme is not. Higher concentrations of serum are required to inhibit the leukocyte enzyme (Janoff, 1972b). Although the situation at present is complicated by the separation of the leukocyte enzyme into three distinct isozymes (Janoff, 1973; Sweetman, et. al., 1973), it would appear logical to assume that the leukocyte elastase(s) and pancreatic elastase are members of the same family of enzymes. The differences in their chemical behavior are probably due to differences in their amino acid sequences at sites removed from the catalytic site.

Elastase Inhibitors. Synthetic inhibitors with a variety of functional groups have been shown to react stoichiometrically with elastase. These include reagents such as diisopropylphosphorofluoridate, p-nitrophenyl diethyl phosphate, tosyl fluoride and phenyl methane sulfonyl fluoride, all of which react with the strongly nucleophilic active site serine residue of serine proteases, including Ser-188 of elastase (Hartley and Shotton, 1971). Brown and Wold (1973 a,b) have recently shown that n-butyl isocyanate also inhibits elastase by reaction at Ser-188. This reagent was not specific for elastase since in addition it inhibited chymotrypsin. Stoichiometric alkylation of the γ-carboxyl group of a glutamic acid residue, tentatively identified as Glu-6, by 1-bromo-4-(2,4-dinitrophenyl) butane-2-one inhibits porcine pancreatic elastase (Visser, et. al., 1971). However, this reagent was incapable of inhibiting the human leukocyte elastase (Janoff, 1969). Since Glu-6

is not near the active site in porcine pancreatic elastase, a precise explanation for this inhibition reaction is unavailable.

V. Peptide Chloromethyl Ketones

Rationale. A few years ago, it appeared to us that peptide chloromethyl ketones were likely candidates for use as elastase inhibitors. These compounds are relatives of Tos-Phe-CH_2Cl (TPCK) and Tos-LysCH_2Cl (TLCK), the active site specific inhibitors of chymotrypsin and trypsin developed by Elliott Shaw and his coworkers (Shaw, 1970). The crystallographic determinations of the binding modes of peptide chloromethyl ketones to chymotrypsin A_γ (Segal et. al., 1971 a,b) and to subtilisin BPN' (Kraut, et. al., 1971; Robertus, et. al., 1972) have provided revealing insights into the interactions of inhibitors with these serine proteases. The inhibitors are bound to the enzyme via a covalent linkage between the imidazole ring of the active site histidine residue and the methylene group of the chloromethyl ketone moiety (see Figure 3 for a comparison of the reaction of a chloromethyl ketone and a substrate with a serine protease). The peptide chain of an extended inhibitor and a section of three residues of the backbone of chymotrypsin and subtilisin form an antiparallel β-sheet structure. In addition, the rates of inactivation of chymotrypsin A_α (Kurachi, et. al., 1973) and subtilisin BPN' (J. C. Powers and J. T. Tippett, unpublished observations) by chloromethyl ketones in solution are dependent upon the interactions between the enzyme and inhibitor both in the region of the primary specificity site and at subsites far removed from the catalytic site.

Synthesis of a series of peptide chloromethyl ketones with an alanine as the P_1 residue (see Figure 4 for the nomenclature) and study of their reactivity with porcine pancreatic elastase produced a series of effective elastase inhibitors (Powers and Tuhy, 1972 and 1973, see also Thompson and Blout, 1973a for similar independent studies). The

Serine Protease

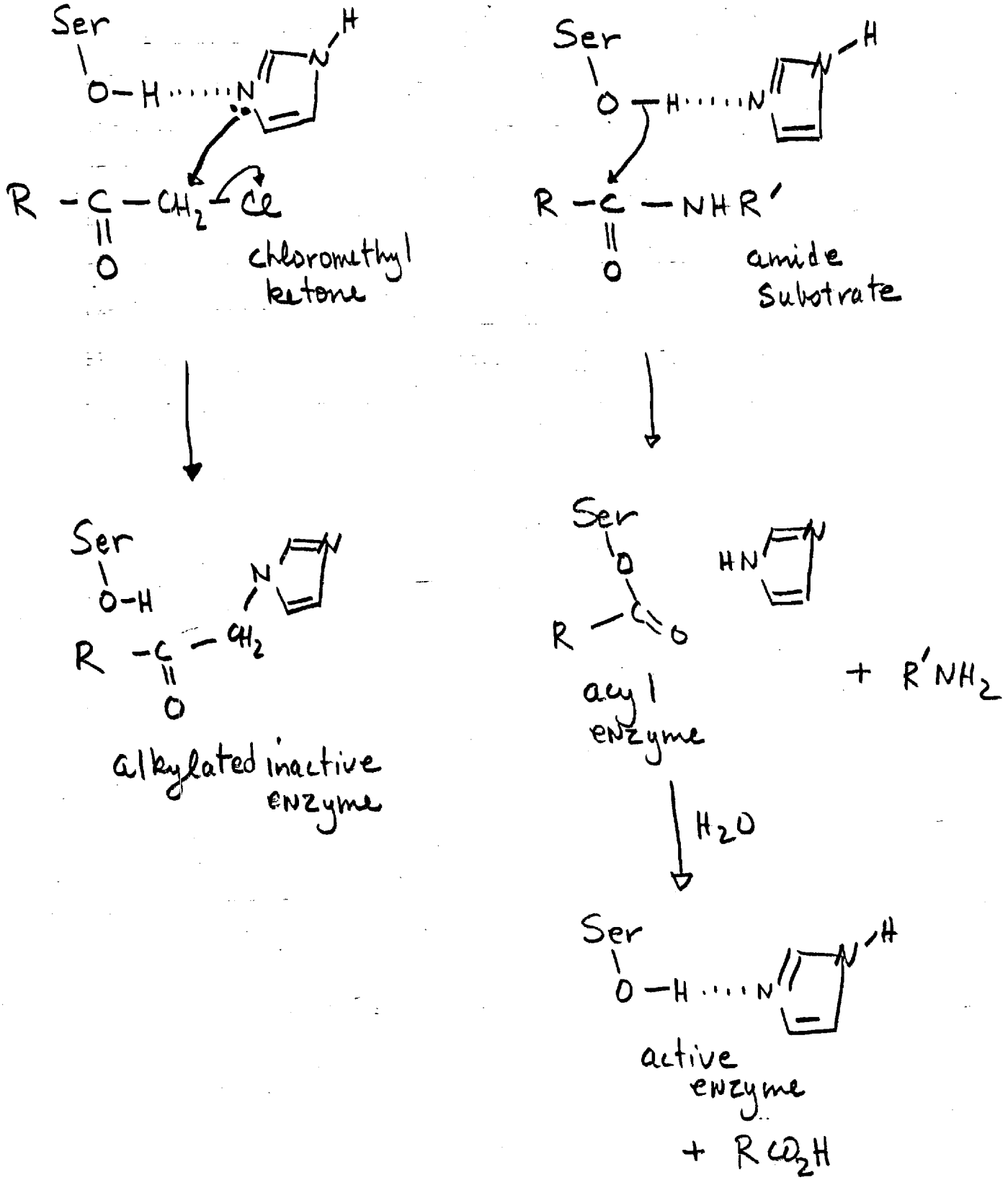


Fig 3 – Comparison of the reaction of a chloromethyl ketone and a substrate with a serine protease

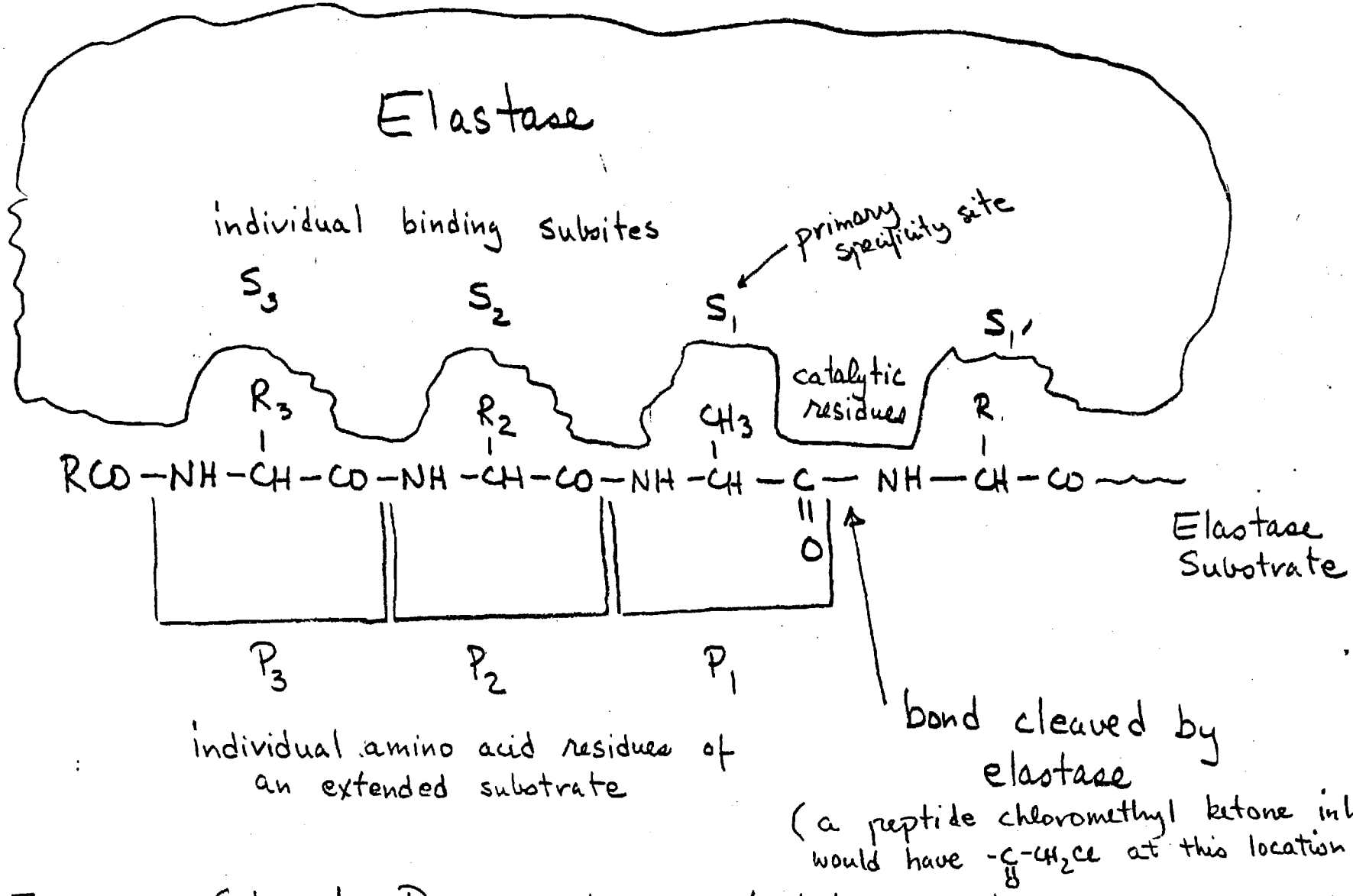


Fig. 4 Schematic Drawing of an extended substrate bound to Elastase.

tetrapeptide chloromethyl ketone, Ac-Ala-Ala-Pro-AlaCH$_2$Cl, was the most effective inhibitor in the series. The inhibitors were very specific for elastase since the homologous serine proteases, chymotrypsin and trypsin, were not significantly inhibited under identical conditions. The tetrapeptide inhibitors were 10 to 80 times more reactive than the tripeptides. This increased reactivity in terms of k_3/K_I was due both to the better binding of the inhibitor to elastase (the K_I of Ac-Ala-Ala-Ala-AlaCH$_2$Cl is three times lower than that of Ac-Ala-Ala-AlaCH$_2$Cl) and to the increased rate of reaction within the bound complex (the k_3 for Ac-Ala-Ala-Ala-AlaCH$_2$Cl is three times higher than that for Ac-Ala-Ala-AlaCH$_2$Cl). Since peptide substrates exhibit a similar reactivity difference between tripeptides and tetrapeptides, both inhibitors and substrates are probably interacting with the same extended binding site in elastase. Proline and/or leucine are the preferred residues at the P_2 site of an inhibitor (e.g., as in Ac-Ala-Ala-Pro-AlaCH$_2$Cl or Z-Gly-Leu-AlaCH$_2$Cl), Pro at the P_3 site (as in Ac-Pro-Ala-AlaCH$_2$Cl or Ac-Ala-Pro-Ala-AlaCH$_2$Cl) renders an inhibitor incapable of inhibiting elastase. In summary, a series of reactive and specific elastase inhibitors had been prepared and had been used to partially map the individual subsite requirements in order to obtain the optimium inhibitor structure.

Synthetic Results. The first goal of this contract was to prepare large quantities of Ac-Ala-Ala-Ala-AlaCH$_2$Cl and Ac-Ala-Ala-Pro-AlaCH$_2$Cl for distribution to other investigators. This was accomplished using the synthetic scheme listed in Fig. 5 and experimental details are published in Powers and Tuhy (1973).

Distribution. As of October 1975, a total of 37 separate shipments of chloromethyl ketones to almost that many different investigators have been made. Each shipment was made in a mailing tube and a letter

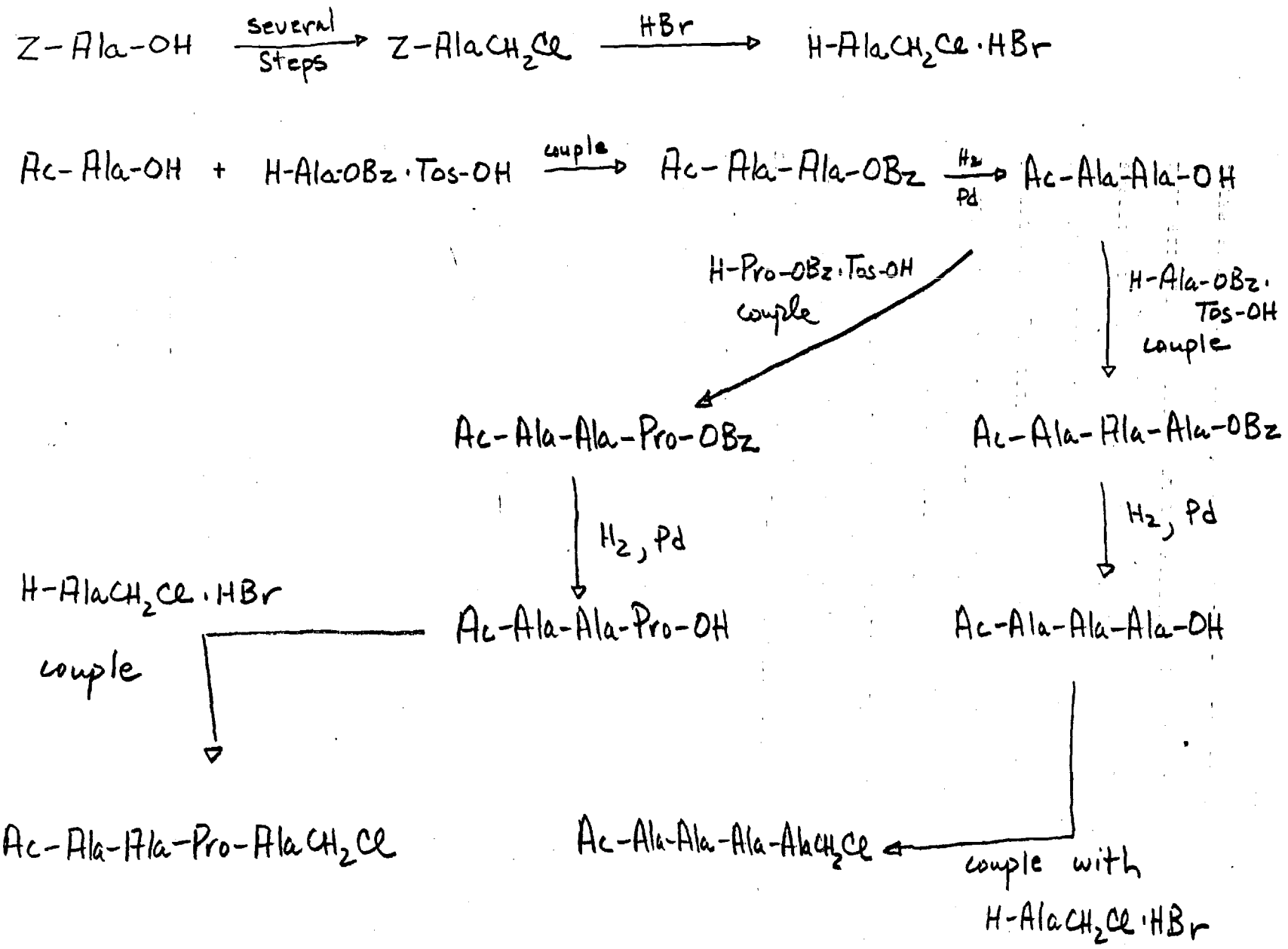


Fig. 5. Synthesis of the chloromethyl ketones Ac-Ala-Ala-Ala-AlaCH$_2$Cl and Ac-Ala-Ala-Pro-AlaCH$_2$Cl.

containing detailed information with literature references was mailed at the same time. In this way, the investigator would be expecting the shipment and would be alerted if it did not arrive. In all cases except one or two, the shipment arrived safely. In the others, a second shipment was made. A list of the investigators and the amounts inhbititors sent are listed in Table I. At this time, we have on hand approximately 7 g of Ac-Ala-Ala-Ala-AlaCH_2Cl and 5.75 g of Ac-Ala-Ala-Pro-AlaCH_2Cl and have shipped 0.94 and 1.28 g respectively.

Inhibition of Human Leukocyte Elastase. The second goal of this contract was to study the inhibition of leukocyte elastase with a series of peptide chloromethyl ketones in order to optimize the inhibitor structure. This has been accomplished using enzyme graciously supplied by Aaron Janoff (Stoney Brook, N.Y.) and is published (Tuhy and Powers, 1975). A copy of that paper is attached as an appendix.

The results indicate that leukocyte elastase reacts less rapidly overall with this group of peptide chloromethyl ketones than does pancreatic elastase, as indicated by smaller values of $k_{obsd}/[I]$ for inhibition. The increase in reactivity toward inhibitors in going from tripeptides to tetrapeptides is smaller in the case of leukocyte elastase, which may indicate differences in the geometry of the extended binding sites and the effect of the individual subsites on the catalytic activity of these two enzymes. The most effective inhibitor for both the human leukocyte and porcine elastase in this series of compounds is Ac-Ala-Ala-Pro-AlaCH_2Cl. Interestingly, its isomer Ac-Ala-Pro-Ala-AlaCH_2Cl is a relatively good inhibitor for the leukocyte enzyme but is not effective toward the pancreatic enzyme, which is strong evidence that the leukocyte and pancreatic enzymes are distinct entities.

A series of inhibitors with varied P_1 residues was recently synthesized and their rates of inhibition measured with the human leu-

kocyte elastase. From the following data, it can be seen that both Ac-Ala-Ala-Pro-ValCH_2Cl and Ac-Ala-Ala-Pro-IleCH_2Cl are more effective inhibitors than Ac-Ala-Ala-Pro-AlaCH_2Cl.

Inhibitor	Relative inhibition rate
Ac-Ala-Ala-Pro-ValCH_2Cl	49
Ac-Ala-Ala-Pro-IleCH_2Cl	40
Ac-Ala-Ala-Pro-AlaCH_2Cl	1
Ac-Ala-Ala-Pro-ThrCH_2Cl	0.5

We have synthesized 2 g of Ac-Ala-Ala-Pro-ValCH_2Cl for distribution, but at present have received no requests for this inhibitor.

Published Studies of other Investigators. Since our initial report of the synthesis of alanyl peptide chloromethyl ketones and their effectiveness in inhibiting porcine elastase, other elastolytic enzymes have also been shown to be inhibited by these compounds. The digestion of human lung tissue and rat aortic tissue by porcine pancreatic elastase or human polymorphonuclear leukocytic elastase is totally inhibited by Ac-Ala-Ala-AlaCH_2Cl *in vitro* (Janoff, 1972). Mosser, *et. al.*, (1973) have recently shown that preincubation of human leukocyte lysosomes with Ac-Ala-Ala-Pro-AlaCH_2Cl depresses the ability of the lysosomal preparation to promote agglutinability by concanavalin A in mouse fibroblasts. In addition, Ac-Ala-Ala-AlaCH_2Cl has proven useful in the location of elastolytic enzymes on disc electrophoresis zymograms; it inhibits the esterolytic activity of three major neutrophil esterases from human polymorphonuclear granules which exhibit elastase-like behavior (Sweetman, *et. al.*, 1973). The chloromethyl ketone inhibitors were used in studies which suggest that PMN elastase plays a role in the digestion of certain bacteria (Janoff and Blondin, 1973 and 1974). The digestion of

TABLE I. Distribution of Chloromethyl Ketones.

	Name	Ac-Ala-Ala-Ala-AlaCH$_2$Cl	Ac-Ala-Ala-Pro-AlaCH$_2$Cl
#1	John A. Pierce, M.D. Pulmonary Disease Division Barnes & Wohl Hospitals 660 South Euclid Avenue Box 8121 St. Louis, Missouri 63110		50 mg 9/13/74
2	Irwin E. Liener, Ph.D. Department of Biochemistry 140 Gortner Laboratory University of Minnesota St. Paul, Minnesota 55101	10 mg	12 mg 9/13/74
3	James O. Harris, M.D. Pulmonary Section Veterans Administration Hospital Gainesville, Florida 32601	11 mg	12 mg 9/13/74
4	Morton Galdston, M.D. New York University Medical Center School of Medicine 550 First Avenue New York, New York 10016	54 mg	52 mg 9/13/74
5	Jerome Klienerman, M.D. Saint Luke's Hospital 11311 Shaker Boulevard Cleveland, Ohio 44104		152 mg 9/13/74
6	Arthur Dannenburg, Ph.D. Dept. Enviromental Medicine School of Hygiene & Public Health The Johns Hopkins University 615 North Wolfe Street Baltimore, Maryland 21205		26 mg 9/13/74
7	Robert Senior, M.D. The Jewish Hospital of St. Louis 216 South Kingshighway St. Louis, Missouri 63110	30 mg 9/13/74	
8	Virginia Richmond, M.D. Virginia Mason Research Center 1000 Seneca Street Seattle, Washington 98101	52 mg	53 mg 9/13/74
9	Ines Mandl, Ph.D. Department of Obsterics & Gynecology College of Physicans & Surgeons Columbia University 630 West 168th Street New York, New York 10032	53 mg	51 mg 9/13/74

TABLE I. (Con't)

10	Daniel Menzel, M.D. Department of Physiology & Pharmaiology Duke University Medical Center Durham, North Carolina 27710	54 mg	105 mg 9/13/74
11	Robert I. Lehrner, M.D. Department of Medicine Center for Health Sciences VCLA Los Angeles, California 90024	11 mg	14 mg 9/19/74
12	S. S. Spicer, Ph.D. Department of Pathology Institute of Pathology Medical University of South Carolina 80 Barre Street Charleston, South Carolina 29401		10 mg 9/13/74
13	Harold Resnick, M.D. National Institute for Occupational Safety & Health P.O. Box 4292 Morgantown, West Virginia 26505	5 mg	9 mg 9/13/74
14	V. B. Hatcher, Ph.D. Montefiore Hospital & Medical Center 111 East 210th Street Bronx, New York 10467	11 mg	11 mg 9/13/74
15	A. Myron Johnson, Ph.D. Department of Pediatrics School of Medicine University of North Carolina Chapel Hill, North Carolina 27514	7 mg	8 mg 9/13/74
16	Thomas Kravis, M.D. Experimental Immunology Division Clinical Medical Sciences Department Naval Medical Research Institute National Naval Medical Center Bethesda, Maryland 20014		12 mg 9/19/74
17	Kjell Ohlsson, M.D. Department of Surgery University of Lund Malmo General Hospital 214 01 Malmo Sweden		133 mg 11/74
18	Phillip Stone, Ph.D. Department of Biochemistry Boston University Medical Center School of Medicine 80 East Concord Street Boston, Massachusetts 02118	207 mg 12/31/74	

TABLE I (Con't)

19	Chin C. Huang, M.D. Biochemistry Research Department Armour Pharmaceutical Company Box 511 Kankakee, Illinois 60901		10 mg 12/31/74
20	Marc H. Dresden, Ph.D. Department of Biochemistry Baylor College of Medicine Texas Medical Center Houston, Texas 77025		10 mg 2/19/75 5.1 mg 6/1/75
21	Isaac Ginsburg, Ph.D. Department of Oral Biology The Hebrew University Hadassah Medical School P.O. Box 1172 Jerusalem, Israel		10 mg 2/19/75
22	Larry Wahl, Ph.D. Laboratory of Developmental Biology & Anomalies National Institute of Dental Research Bethesda, Maryland 20014		10 mg 4/4/75
23	Aaron Janoff, Ph.D. Department of Pathology Health Sciences Center State University of New York, Stony Brook Stony Brook, New York 11790	100 mg	100 mg 4/15/75
24	Yoshiko Narahashi, Ph.D. Polymer Chemistry Lab The Institute of Physical & Chemical Research 2-1, Hirosawa, Wakoshi Saitama, JAPAN	11 mg (and 11 mg Ac-Ala-Ala-AlaCH$_2$Cl)	18 mg 4/24/75
25	Victor Hatcher, Ph.D. Montefiore Hospital & Medical Center Albert Einstein College of Medicine 111 East 210th Street Bronx, New York 10467	22 mg	21 mg 5/8/75
26	Robert A. Clark, M.D. School of Medicine Department of Medicine RM-16 University of Washington Seattle, Washington 98195 (Second shipment)	53 mg 53 mg	50 mg 6/2/75 50 mg 9/16/75
27	J. C. Taylor, Ph.D. Scripps Clinic ¢ Research Foundation Department of Microbiology 476 Prospect Street La Jolla, California 92037		51 mg 6/20/75

TABLE I. (Con't)

28	Bruce Sloan, Ph.D. Pulmonary Disease Section Albert Einstein Medical Center York & Tabor Roads Philadelphia, Pennsylvania 19141	102 mg	100 mg 6/20/75
29	Edward Bittar, M.D. Section 6205 Memorial Sloan Kettering Cancer Center 1275 York Avenue New York, New York 10021	12.1 mg	10 mg 8/25/75
30	John Gleisner, Ph.D. Virginia Mason Research Center 1000 Seneca Street Seattle, Washington 98101	11 mg	10 mg 8/26/75
31	John Aronson, Ph.D. Department of Medicine Hospital of the University of Pennsylvania 3400 Spruce Street Philadelphia, Pennsylvania 19104		5.6 mg 8/26/75
32	Alvin Davis, M.D. Infectious Disease Section Veterans Administration Hospital 5901 East 7th Street Long Beach, California 90801	6.5 mg	6.6 mg 9/16/75
33	Jerry Dolovich, M.D. Department of Pediatrics McMaster University 1200 Main Street, West Hamilton, Ontario CANADA, L8S 4J9		6.3 mg 8/26/75
34	A. C. Allison, M.D. Clinical Research Centre Division of Cell Pathology Watford Road Harrow, Middlesex England HA1 3UJ	61 mg	50 mg 9/16/75
35	Friedrich Kueppers, M.D. Thoracic Diseases & Internal Medicine Mayo Clinic Rochester, Minnesota 55901		50 mg 9/29/75
		936.6 mg	1283.6 mg

articular cartilage proteglycan by granules and human neutrophil elastase is stopped by treatment with Ac-Ala-Ala-AlaCH$_2$Cl and Ac-Ala-Ala-Pro-AlaCH$_2$Cl (Malemud and Janoff, 1975).

A human pancreatic enzyme, protease E, is inhibited by a number of alanyl chloromethyl ketones (Mallory and Travis, 1975). Cathepsin B was also inhibited by Ac-Ala-Ala-Ala-AlaCH$_2$Cl and Ac-Ala-Ala-Pro-AlaCH$_2$Cl (Barrett, 1973; Starkey and Barrett, 1973; Burleigh et. al., 1974).

VI. Peptide Carbazates.

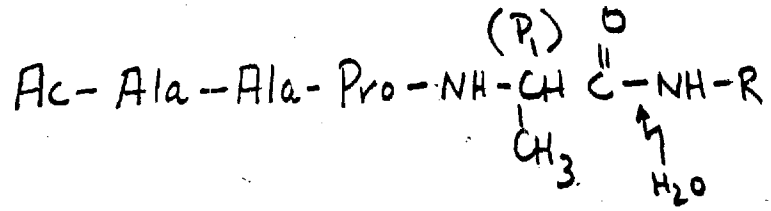
Rationale. Peptide carbazates can be considered to be analogs of peptide substrates in which the α-carbon atom of the P_1 residue has been replaced by a nitrogen atom (see Figure 6). They would thus be expected to acylate a serine protease with the appropriate specificity in much the same fashion as simple synthetic substrates. The acylated enzymes should be considerably more stable toward deacylation than a normal "acyl enzyme" due to the influence of the adjacent nitrogen atom. The carbazate, p-nitrophenyl N^2-acetyl-N^1-benzylcarbazate, has been used as an active site titrant for chymotrypsin (Elmore and Smyth, 1968). The carbazate Ac-Ala-NHN($CH_2C_6H_5$)CO-ONp has been prepared and shown to acylate chymotrypsin irreversibly (half life for reactivation >52 hrs) (Powers and Carroll, unpublished results).

The third goal of this contract was to synthesize peptide carbazates as elastase inhibitors and study their effectiveness.

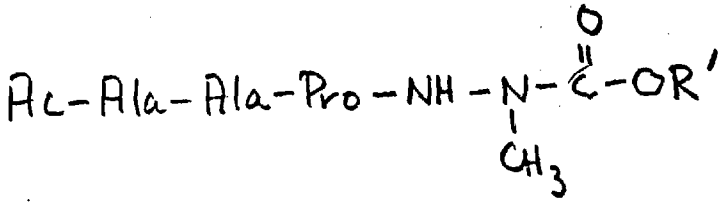
Synthetic Results and Distribution. Three carbazates have been synthesized by the methods outlined in Fig. 7. One carbazate, Ac-Ala-Ala-Mec-ONp, has been prepared in sufficient quantities (ca. 1 g) for distribution. At present, this carbazate has been sent to the following three investigators:

Dr. Aaron Janoff
Department of Pathology
Health Sciences Center
State University of New York, Stony Brook
Stony Brook, New York 11790 51 mg 7/18/75

Fig. 6 Comparison of the Reaction of Elastase with Substrates and Carbazates

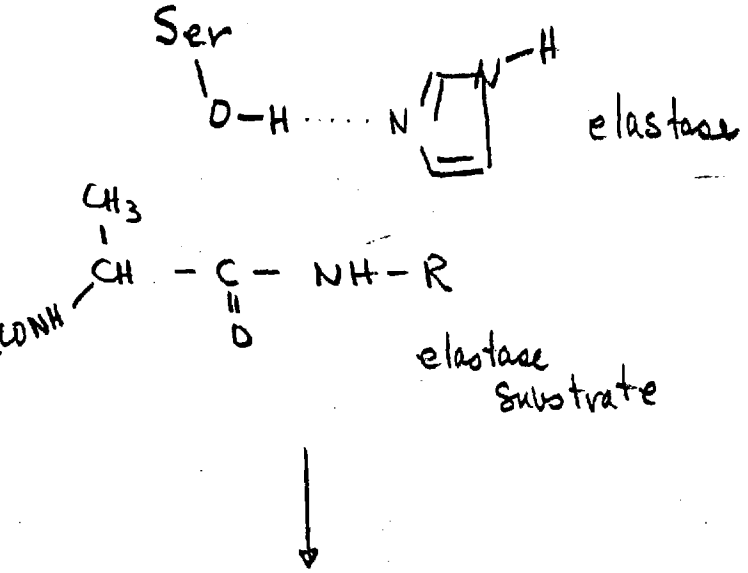


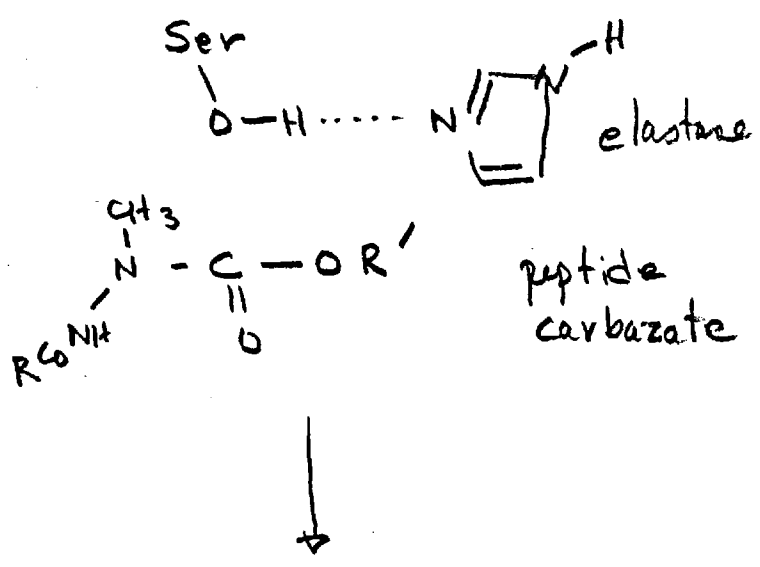
good elastase Substrate

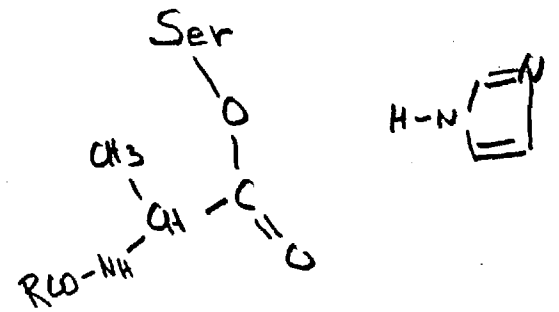


peptide carbazate analogous to a good substrate

R'OH = HONp, CF_3CH_2OH or any other electron deficient alcohol.



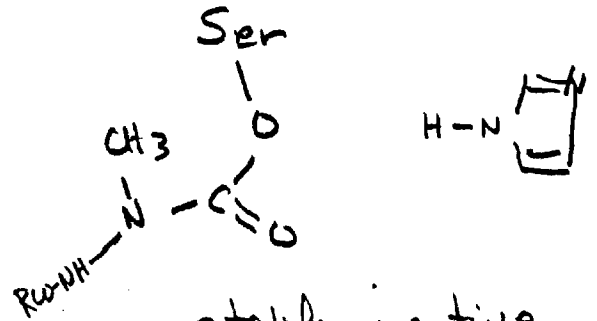




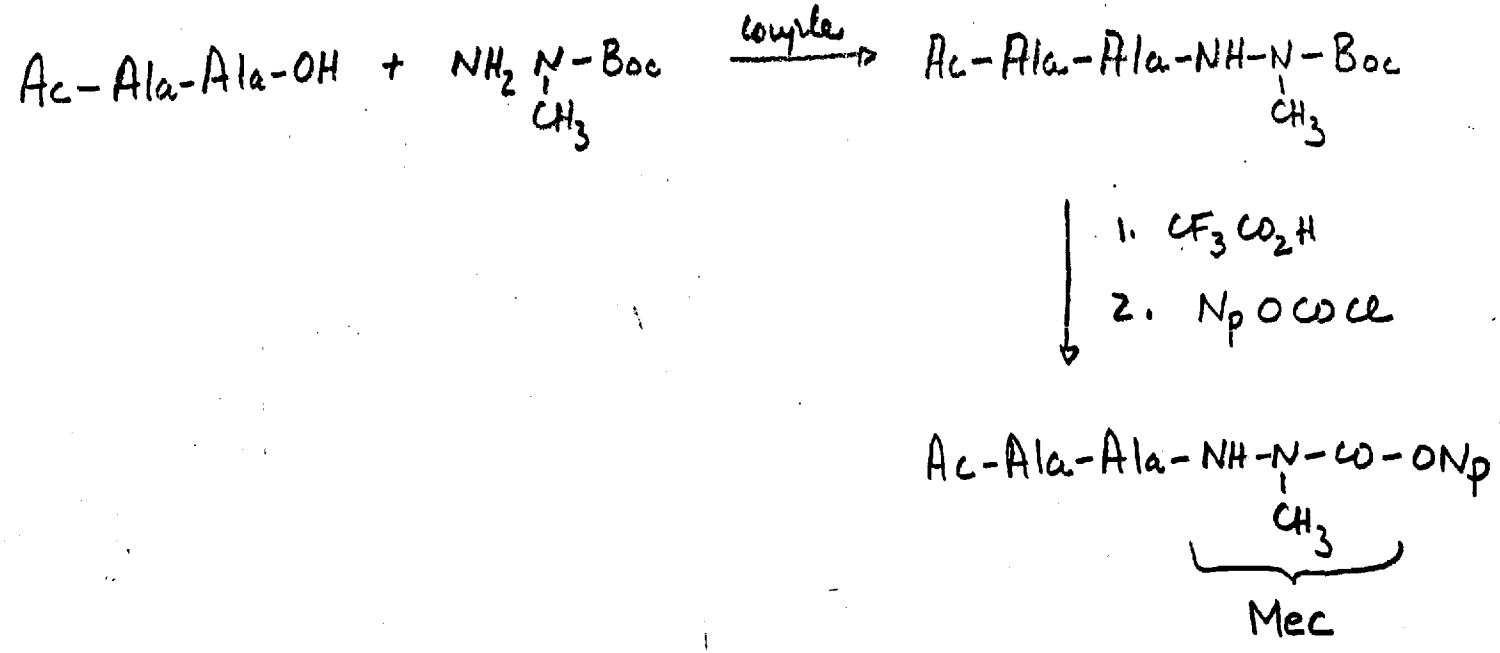
Acyl enzyme

$\downarrow H_2O$

active enzyme + product



stable inactive elastase derivative



Ac-Ala-Ala-Ala-OH $\xrightarrow{\text{similarly}}$ Ac-Ala-Ala-Ala-Mec-ONp

Ac-Ala-Ala-Pro-OH $\xrightarrow{\text{similarly}}$ Ac-Ala-Ala-Pro-Mec-ONp

Fig. 7. Synthesis of Peptide Carbazates.

Dr. A. J. Barrett
Strangeways Research Laboratory
Wort's Causeway
Cambridge. CB1 4RN — Will be mailed shortly

Dr. Ohlsson
Department of Surgery
University of Lund
Malmo General Hospital
214 01 Malmo Sweden — Will be mailed shortly

Inhibition of Human Leukocyte Elastase. The dipeptide carbazate Ac-Ala-Mec-ONp(Mec = 2-methyl carbazoic acid) does not react with human leukocyte elastase, an observation that is not suprising in view of the requirement of elastase for an extended peptide chain in a substrate or inhibitor. Both the tetrapeptide and the tripeptide carbazates react with elastase at pH 6.0 to give a burst of 4-nitrophenol within a few seconds. Presumably, a carbazyl enzyme is formed as the other product. The inactive enzyme thus formed, however, regains activity gradually as the carbazyl group is hydrolyzed slowly from the enzyme. The rates of reactivation have not yet been accurately measured, but the $t_{\frac{1}{2}}$ is on the order of 15 min at pH 6.0. The reactivation rate does not seem to be affected by more than twofold when the structure of the carbazate is altered. Thus, it appears carbazates will be useful only as temporary inhibitors of human leukocyte elastase.

Although Ac-Ala-Ala-Mec-ONp is not a useful inhibitor, it can be used as an active site titrant. The release of 4-nitrophenol is stoichiometric, and the deacylation rate, although not negligible, is much slower than the initial acylation. Although Ac-Ala-Ala-Mec-ONp does react with chymotrypsin, it can be used under the appropriate conditions to titrate elastase in the presence of chymotrypsin.

VII. Results and Conclusions

In conclusion, we have clearly demonstrated that peptide chloromethyl ketones are valuable active-site directed inhibitors of human leukocyte

elastase and that peptide carbazates are useful active-site titrants for elastase. We have distributed these inhibitors to thirty-five other investigators, several of which have initiated in vivo studies with chloromethyl ketone inhibitors. They have proved to be useful for elucidating many of the molecular pathways by which human leukocyte elastase performs its physiologic and pathologic functions. In the future, these or related inhibitors should prove to be useful for the treatment of pulmonary emphysema, arthritis and other diseases that involve elastic tissue destruction by elastolytic proteases.

VIII. References

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IX. Personnel Associated with Project

James C. Powers, Ph.D.	Project leader
James R. Boone, Ph.D.	Postdoctorial Research Associate
David L. Carroll, B.S.	Predoctorial Research Associate

X. Publications

"Inhibition of Human Leukocyte Elastase By Peptide Chloromethyl Ketones", P. M. Tuhy and J. C. Powers, FEBS LETTERS, 50, 359 (1975).

"Synthetic Active Site-Directed Inhibitors of Elastolytic Proteases", J. C. Powers, D. L. Carroll and P. M. Tuhy, Ann. N.Y. Acad. Sci., 256, 420 (1975).

Appendix

Copies of both the above papers are attached.